ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF MICROBES IN BIOFERTILIZER

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My opinion this thesis is sufficient in terms of scope and
quality for the award of the degree of

...."

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A thesis submitted in fulfillment
of the requirements for the award of the degree of
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DECLARATION

I declare that this thesis entitled "**Isolation, characterization and identification of microbes in biofertilizer**" is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree."

Signature : ....

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Date : April 2008

DEDICATION

Special Dedication to my family members that always love me,
My friends, my fellow colleague
and all faculty members

For all your care, support and believe in me.

Sincerely
Mohd Farid B Ishak

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ABSTRACT

Biofertilizer has been identified as an alternative to chemical fertilizer to increase soil fertility and crop production in sustainable farming. The objectives of this study are to isolate microorganism in biofertilizer and to study the characteristic of microbe that can enhance the uptake of macronutrient which is nitrogen (N), potassium (K) and phosphate (P). This study involves with isolation of microbes in biofertilizer, which can enhance the uptake of macronutrient. After that, the microbes are characterized using three staining methods which are gram staining, acid-fast staining and spore staining also the catalase test to determine the type of microbes. The morphology of microbes can be observed after all the staining process is done by observing the colour change and the shape of each microbe. In the nut shell, characterization of this microbe can be done by doing three steps of staining methods and catalase test.

ABSTRAK

Baja bio-organik telah dikenalpasti sebagai salah satu baja alternatif untuk mengantikan baja kimia untuk meningkatkan kesuburan tanah dan hasilan tanaman. Objektif kajian ini adalah untuk mengasingkan mikroorganisma di dalam baja bio-organik, dan untuk mengkaji sifat mikroorganisma yang dapat menaikkan kadar pengambilan nutrien makro iaitu nitrogen (N), kalsium (K) dan juga fosforus (P). Kajian ini melibatkan pengasingan mikroorganisma di dalam baja bio organik untuk mendapatkan mikroorganisma yang dapat menaikkan kadar pengambilan nutrien makro. Selepas itu mikroorganisma itu di cirikan dengan menjalankan tiga jenis proses pewarnaan iaitu pewarnaan gram, pewarnaan keasidan dan pewarnaan spora dan juga ujian terhadap pemangkin untuk mengenal pasti jenis mikroorganisma tersebut. Morfologi setiap mikroorganisma tersebut dapat di ketahui selepas kesemua proses pewarnaan dijalankan dengan melihat perubahan pada warna serta bentuk setiap mikroorganisma. Secara kesimpulannya, pengasingan mikroorganisma ini dapat dijalankan dengan melakukan tiga jenis proses pewarnaan ini dan juga ujian terhadap pemangkin.

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AAS	-	atomic absorption spectrometers
AMF	-	arbuscular mycorrhizal fungus
BNF	-	Biological Nitrogen Fixation
CO_2	-	carbon dioxides
EMB	-	Eosin-Methylene Blue
g	-	gram
h	-	hour
H_2O_2	-	hydrogen peroxides
H_2S	-	hydrogen sulfide
H_2SO_4	-	sulfuric acids
HNO_3	-	nitrite acids
K	-	potassium
KSB	-	potassium solubilizing bacteria
L	-	liter
M	-	Mole
mg	-	miligram
min	-	minute
mL	-	milliliter
mM	-	milimole
MPN	-	Most Probable Number
N	-	nitrogen
P	-	phosphate
PGPR	-	plant growth promoting rhizobacteria
PSB	-	phosphate solubilizing bacteria
rpm	-	rotation per minutes

S	-	sulfur
sec	-	seconds
TOC	-	Total Organic Carbon
TSA	-	Tryptic Soy Agar

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Microorganism can live in everywhere, in the air, water and soil, and in the body of human beings and other creatures. Society benefits from microorganisms in many ways. They are necessary for the production of bread, cheese, bear, antibiotics, vaccines, vitamins, enzymes, and another important product. Microorganisms are indispensable components of our ecosystem. They make possible the carbon, oxygen, nitrogen, and sulfur cycles that take place in terrestrial and aquatic system, and are a source of nutrients at the base of all ecological food chains and webs. Their benefits are enormous in the field of agriculture (biofertilizer) and in bioremediation of polluted resources. In the field of agriculture, microbe that have a function as a decomposers will breakdown the organic material, form humus and unlock the useful nutrients (N, P, K, S, trace elements etc) and made them available to plants (Han *et al.,* 2005)

1.2 Problem Statement

Nowadays most farmer use chemical fertilizer as a source of nutrient for their crop but chemical fertilizer gives side impact to the soil itself. Normally chemical fertilizer contain chemical sustains that are harmful to the soil if it was use for long term.

Biofertilizer has been identified as an alternative to chemical fertilizer to increase soil fertility and crop production in sustainable farming. The utilization of microbial products has several advantages over conventional chemicals for agricultural purposes: (i) microbial products are considered safer than many of the chemicals now in use; (ii) neither toxic substances nor microbes themselves will be accumulated in the food chain; (iii) self-replication of microbes circumvents the need for repeated application; (iv) target organisms seldom develop resistance as is the case when chemical agents are used to eliminate the pests harmful to plant growth; and (v) properly developed biocontrol agents are not considered harmful to ecological processes or the environment (Weller, 1988; Gloud, 1990; Shen, 1997).

Biofertilizers are products containing living cells of different types of microorganisms, which have an ability to convert nutritionally important elements from unavailable to available form through biological processes (Hegde *et al*., 1999; Vessey, 2003). In recent years, biofertilizers have emerged as an important component of the integrated nutrient supply system and hold a great promise to improve crop yields through environmentally better nutrient supplies. However, the application of microbial fertilizers in practice, somehow, has not achieved constant effects. The mechanisms and interactions among these microbes still are not well understood, especially in real applications (Wu *et al.*, 2004).

By those problems, the outcome of these research will encounter the problem occur and make the fertilizer is environmental friendly. The result will enhance the biofertilizer in term of nutrient uptake which is nitrogen (N), potassium (K) and phosphate (P).

1.3 Objectives of Study

i) To isolate microorganisms in biofertilizer.

ii) To study the characteristic of microbes to enhance the quality of biofertilizer.

iii) To determine microbes that can provide optimum of macronutrient which is nitrogen (N), potassium (K) and phosphate (P) to the plant.

1.4 Scope of Study

i) Isolate microbe in biofertilizer by using isolation method.

ii) Identify the microbe that can give optimum uptake of macronutrient for plant growth.

CHAPTER 2

LITERATURE REVIEW

2.1 Nitrogen fixer (N-fixer)

Biological Nitrogen Fixation (BNF) occurs when atmospheric nitrogen is converted to ammonia by a pair of bacterial enzymes called nitrogenase (Postgate, 1998). The formula for BNF is:

$$N_2 + 8H^+ + 8e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16\ P_i$$

Although ammonia (NH_3) is the direct product of this reaction, it is quickly protonated into ammonium (NH_4^+). In free-living diazotrophs, the nitrogenase-generated ammonium is assimilated into glutamate through the glutamine synthetase/glutamate synthase pathway.

In most bacteria, the nitrogenase enzymes are very susceptible to destruction by oxygen (and many bacteria cease production of the enzyme in the presence of oxygen) (Postgate, 1998). Low oxygen tension is achieved by different bacteria by: living in anaerobic conditions, respiring to draw down oxygen levels, or binding the oxygen with a protein (Postgate, 1998; Smil, 2000).

Nitrogen can also be artificially fixed for use in fertilizer, explosives, or in other products. This artificial fertilizer production has achieved such scale that it is now the largest source of fixed nitrogen in the earth's ecosystem (Laplaza *et al*, 1995).

2.2 Phosphate and Potassium solubilizer (P-solubilizer and K-solubilizer)

The use of plant growth promoting rhizobacteria (PGPR), including phosphate and potassium solubilizing bacteria (PSB and KSB), as biofertilizers has become of interest in many agriculture country as significant areas of cultivated soils are deficient in soil available nutrient which is P and K (Xie, 1998). P and K are major essential macronutrients for plant growth and development and hence they are commonly added as fertilizer to optimize yield. PSB have been used to convert insoluble rock P material into soluble forms available for plant growth (Nahas *et al,* 1990; Bojinova *et al,* 1997). This conversion is through acidification, chelation and exchange reactions and produce, in the periplasm, strong organic acids, which have become indicators for routine isolation and selection procedures of PSB (Illmer, 1995). *Bacillus megaterium* is known for its ability to solubilize rock P material (Schilling *et al,* 1998). On the other hand, KSB are able to solubilize rock K mineral powder, such as micas, illite and orthoclases, through production and excretion of organic acids (Friedrich *et al,* 1991; Ullman *et al*, 1996). It has been shown that KSB, such as *Bacillus mucilaginous*, increased K availability in soils and increased mineral content in the plant (Sheng *et al,* 2002). A combination of application of rock P and K materials with co- inoculation of bacteria that solubilize them might provide a faster and continuous supply of P and K for optimal plant growth. However little is known about the combined effects of rock materials and co-inoculation of PSB and KSB on mineral availability in soils, mineral content and growth of eggplant. The present work reported the synergistic effects of soil fertilization with rock P and K materials and co-inoculation with PSB and KSB on the improvement of P and K uptake and growth of eggplant grown under limited P and K soil under greenhouse conditions (Hans *et al*, 2005).

2.3 Biofertilizer

The macro and micro-nutrient components in biofertilizer are so integrated that they effectively interact to provide and enrich the nutrient base of the soil in the most efficient and effective manner, yet being most economic. Thus, apart from replenishing the soil and providing the crop with macro-nutrients like nitrogen, phosphate and potash, biofertilizer also enriches the soil with essential micro-nutrients that include zinc, boron, magnesium, and molybdenum among others. Moreover, biofertilizer also increases the organic matter content of the soil along with the major and minor nutrients which are also organic based in biofertilizer itself.

The combined interaction of the nutrient ingredients in biofertilizer integrates the soil with the full range of nutrients within a relatively short time, and their effects last longer for the standing crop to benefit directly. By optimally utilizing these nutrients from the soil, crop productivity in the biofertilizer treated plots greatly increases as reflected in the high yields and quality of the crops.

2.4 Advantages and Disadvantages of Microorganisms

2.4.1 Advantages / benefits

Microorganism can live in everywhere, in the air, water and soil, and in the body of human beings and other creatures. Society benefits from microorganisms in many ways. They are necessary for the production of bread, cheese, bear, antibiotics, vaccines, vitamins, enzymes, and another important product. Microorganisms are indispensable components of our ecosystem. They make possible the carbon, oxygen, nitrogen, and sulfur cycles that take place in terrestrial and aquatic system, and are a source of nutrients at the base of all ecological food chains and webs. Their benefits are enormous in the field of agriculture (biofertilizer) and in bioremediation of polluted resources.

2.4.1.1 Fixation of atmospheric nitrogen

In the field of agriculture atmospheric nitrogen is fixed by *Rhizobium* (rhizobia) bacteria in the nodules of legumes in the form of complex compounds of nitrogen. Some bacteria are free-living organisms and have the capacity to absorb atmospheric N_2 to synthesis organic nitrogenous compounds. These organisms (non-symbiotic nitrogen fixing organisms) are bacteria, algae and fungi and are aerobic, anaerobic, heterotrophic, autotrophic and photosynthetic.

2.4.1.2 Mineralization of organic nitrogen compounds

Mineralization of nitrogen is the conversion of organic form nitrogen to inorganic / mineral form of nitrogen such as NH^{4+}**,** NO^{2-} and NO^{3-}. It takes place in three steps which is aminization, ammonification and nitrification.

i) **Aminization:** the protein breaks down to yield amines, amino acids, carbon dioxide, energy and other products. This process is brought about by some heterotrophic soil microorganisms. They are mostly bacteria and fungi and possibly actinomycetes.

ii) **Ammonification:** in this case the amines and amino acids released by aminization process are converted to ammonia, NH^3. The conversion is caused by another group of heterotrophic of soil microorganisms such as bacteria, fungi and actinomycetes.

iii) Nitrification: this process is completed in two steps which is in the first step nitrite (NO^{2-}) are form and in the second step NO^{3-} are formed. These two steps are caused by two enzymes, dehydrogenase and oxidase, secreted obligate autotrophic bacteria such as nitrosomonas, nitrosococcus, nitrosocystis, nitrosospira and nitrosogloea.

From the above it can be concluded that life within soil exceeds the life above the soil in terms of numbers of living organisms and total metabolic activity (Thomson *et al.,* 1978). Soil is a unique environment as it contains a vast array of bacteria, actinomycetes, fungi, algae and protozoa, which are important group of micro flora in soils. They are unicellular, of numerous genera and perform vast variety of specialized functions. The important bacteria belong to symbiotic-N-fixation, no symbiotic-N-fixation, aminization, ammonification and nitrification, P-solubilizer, K-solubilizer, S-oxidizers and H_2S oxidizers. Some important bacteria from plant nutrition point of view are listed in Table 2.1

2.4.1.3 Decomposers

Decomposers (fungi, bacteria and actinomycetes) breakdown the organic material, form humus and unlock the useful nutrients (N, P, K, S, trace elements etc) and made them available to plants. Phosphate solubilizers (fungi and bacteria) secrete organic acids and help in solubilization of insoluble P-compound in soil. Mycorrhizal fungi have been reported to mobilized P and other nutrients in normal as well as saline soils. Some bacteria live in association with roots of cereals such as rice, sugarcane and grasses, and produce phyto hormones to enhance plant growth.

Some microbes act as biological control of pathogens, due to liberation of antibiotic and other compounds. The bacteria which affect insects are broadly classified as spore-formers and non-spore-formers. The spore formers include milky disease organisms, *Bacillus popilliae, B. lentimorbus* and facultative groups such as *B. sphaericus* and *B. thuringiensis*. The non-spore-formers include *serratia, pseudomonas, aerobacter* and *streptococcus*. The spore forming bacteria are promising organisms for microbial control (K.R. Dadarwal).

Table 2.1: Important bacteria for plant

No.	NAME OF BACTERIA	FUNCTION
1	*Rhizobium sp*	symbiotic N fixation
2	*Bradyrhizobium*	symbiotic N fixation
3	*Cyanobacteria* (BGA)	biological N fixation
4	*Azotobacter sp*	biological N fixation
5	*Azospirillum sp*	biological N fixation
6	*Nitrobacter sp*	nitrification
7	*Nitrosomonas sp*	nitrification
8	*Pseudomonas*	denitrification
9	*Bacillus sp*	denitrification
10	*Paracoccus*	denitrification
11	*Thiobacillus*	denitrification
12	*T. thioparus*	denitrification
13	*Bacillus megaterium*	P solubilizer
14	*B. circulans*	P solubilizer
15	*B. subtilis*	P solubilizer
16	*Pseudomonas straita*	P solubilizer
17	*P. rathonis*	P solubilizer
18	*Escherichia freundla*	P solubilizer
19	*Thiobacillus thioxidans*	oxidizes S
20	*T. thioparus*	oxidizes S
21	*T. copraliticus*	oxidizes S
22	*T. ferrooxidans*	oxidizes S
23	*Beggiatoa*	oxidizes H_2S to S
24	*Thiothrix*	oxidizes H_2S to S

2.4.2 Disadvantages / harmful effects

Microorganisms also had harmed humus and disrupted society since the beginning of recorded history. Microbial diseases undoubtedly played a major role in historical events such as the decline of the Roman Empire and the conquest of the New World. In the year 1347, plague or Black Death stuck Europe with brutal force. By 1351 the plague had killed 1/3 of the population (about 25 million people). Over the next 80 years, the disease stuck again and again, eventually wiping out 75% of the European population. Some historians believe that this disaster changed European culture and prepared the way for the Renaissance. Today, the struggle by microbiologists and others against killers like AIDS and malaria continues (Prescott *et al.*).

The pollution caused by solid city wastes, sewage water and industrial effluent and sludge (solid waste) have played havoc in the developed countries where it is being continuously controlled and the pollution is said to be to a lesser extent and to the largest extent in the developing countries where the problem has multiplied and it is beyond their financial as well as technical resources to control it.

CHAPTER 3

METHODOLOGY

3.1 Isolation of Microbe

3.1.1 Media and reagents.

Specific media broth was used for initial cultivation microbe from biofertilizer. After isolation, microbes strains were routinely cultured using nutrient agar. Microbes were assayed in own selective medium agar that can determine microbe for uptake macronutrient which is nitrogen, phosphate or potassium that has been shown in Table 3.1.

3.1.2 Biofertilizer

Single biofertilizer slurry consisting of 1 g of biofertilizer and 9 mL of 10 mM phosphate buffer (pH 6.8) was used as a common inoculum source for most probable number (MPN) assays. The phosphate buffer was identical to the buffer used in culture medium. After being shaken for 30 min at 30ºC (at 225 rpm on a rotary shaker), the slurry was diluted by transferring 1 mL to 9 mL of sterile phosphate buffer (John *et al*, 1996).